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Absorption and distribution of chitosan in mice after oral administration

Lintao Zeng [a,b], Caiqin Qin [a,*], Wei Wang [b], Weilin Chi [b], Wei Li [a]

[a] *Laboratory for Natural Polysaccharides, Xiaogan University, Xiaogan 432000, China*
[b] *Department of Chemistry, Central China Normal University, Wuhan 430079, China*

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Abstract

Four chitosan samples with different molecular weight M_w and the degree of deacetylation DD (HCS 7.60×10^5 and 85.5%, MCS 3.27×10^4 and 85.2%, COS 0.99×10^3 and 85.7%, WSC 3.91×10^4 and 52.6%) were prepared, and labeled by fluorescein isothiocyanate. These labeled samples were used to investigate the absorption and distribution in mice after oral administration. The results indicated that the absorption and distribution of chitosan was significantly influenced by its M_w and water-solubility. The absorption of chitosan molecules increased with the decrease of the M_w and the increase of the water-solubility. The absorbed chitosan molecules were distributed to all tested organs such as liver, kidney, spleen, thymus, heart and lung. The chitooligomer molecules were easily absorbed and metabolized. The absorbed chitosan molecules from water-soluble WSC in all tested tissues maintained high concentration for a long period. The results suggest that different chitosan may be employed for different functional food.

Keywords: Chitosan; Molecular weight; Water-solubility; Intestinal absorption; Organ distribution

1. Introduction

Chitosan is a linear heteropolysaccharide composed of β-1–4-linked D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) with various compositions of these two monomers, available largely in the exoskeletons of shellfish and insects. The US Food and Drug Administration approved chitosan as a feed additive in 1983. Chitosan has attracted tremendous attention as a potentially important renewable agricultural resource, and has been widely applied in the fields of agriculture, medicine, pharmaceuticals, functional food, environmental protection and biotechnology in the last 20 years (Dodane & Vilivalam, 1998; Harish Prashanth & Tharanathan, 2007; Hejazi & Amiji, 2003; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli, 1996; Thanou, Verhoef, & Junginger, 2001).

Chitosan has many functions such as antitumor activity (Jeon & Kim, 2002; Qin, Du, Xiao, & Li, 2002; Suzuki et al., 1986; Tokoro et al., 1988), cholesterol-lowering effect (Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000; Ormrod, Holmes, & Miller, 1998), immuno-enhancing effect (Peluso et al., 1994), antidiabetic effect (Hayashi & Ito, 2002), wound healing effect (Porporatto, Bianco, Riera, & Correa, 2003), antifungal activity and antimicrobial activity (Qin et al., 2006). Although numerous literatures are available on the aforementioned biological activities, the relationships of these activities with molecular weight and water-solubility of chitosan deserve to be investigated. It can be easily hypothesized that the biological properties of chitosan may be closely related to the molecular weight and water-solubility. As a preliminary study, in vivo absorption phenomena of different chitosan were investigated.

* Corresponding author.
E-mail address: qincaiqin@yahoo.com (C. Qin).

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2. Experimental

2.1. Materials

Crude chitosan was supplied by Golden-shell Biochemical Co., LTD (China). The crude chitosan was purified by HCl and NaOH to get HCS (Qin et al., 2002). Fluorescein isothiocyanate (FITC) was purchased from Sigma Chemical Co. (USA). Other reagents were of analytical grade. Kunming strain female mice (4 weeks old) weighing 20–24 g were purchased from Hubei Experimental Animal Center (China). The UF membranes (OSOO1C11, OMEGA) with NMWL 10 kDa, 5 kDa and 1 kDa were purchased from PallFiltron Corporation (USA).

2.2. Preparation of chitosans with different molecular weights

Fifty gram of crude chitosan was completely dissolved in 1000 ml 2% (v/v) acetic acid, the solution was placed in a water bath at 48 °C and 2.0 g cellulase was added to initiate the reaction.

After 2 h, half of the reaction mixture was taken out, boiled for 10 min to denature the enzyme, and filtered. The UF membrane of 10 kDa was used to separate out the product. The fraction with higher M_w was neutralized with 10% NaOH to pH 9. The precipitate was washed thoroughly with distilled water and ethanol. The sample MCS was collected after drying over phosphorus pentoxide *in vacuum*.

After 8 h, the left reaction mixture passed UF membrane of 5 kDa and 1 kDa to separate. The filtrate was concentrated by rotary evaporator, and neutralized to pH 9 and precipitated by adding ethanol. The precipitate was washed thoroughly with ethanol and collected after drying over phosphorus pentoxide *in vacuum* to gain sample COS.

2.3. Preparation of water-soluble chitosan

MCS (4.2 g) was dissolved in 120 ml 1% acetic acid solution. The solution was diluted with 100 ml absolute alcohol under stirring, and acetic anhydride (2.5 ml) was added to the solution (Qin et al., 2006). After stirring the solution at 20 °C for 12 h, the reaction was ended by adding 4% NaOH until the pH was 9. The solution was concentrated to about 1/5 with a rotary evaporator under diminished pressure, and precipitate by adding ethanol. The precipitate was repeatedly washed with ethanol. The product was dried over P_2O_5 in vacuum at room temperature for 24 h to obtain sample the product WSC.

2.4. Measurement of M_w

The weight average molecular weight (M_w) of samples was measured by a gel permeation chromatography (GPC). GPC system incorporated a TSP P1000 instrument. Two columns in series (TSK G5000-PW and TSK G3000-PW) were used. The eluent was 0.2 M CH_3COOH/0.1 M CH_3COONa. The flow rate was maintained at 1.0 ml min^{-1}. The temperature of the columns was maintained at 30 °C. The eluent was monitored by a RI 150 refractive index detector. The sample concentration was ca.0.4% (w/v). The standards used to calibrate the column were TOSOH pullulan. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

2.5. Estimation of the water-solubility

The pH dependence of water-solubility of chitosan was evaluated from turbidity. Chitosan (0.3 g) was completely dissolved in 100 ml 1% acetic acid. The pH of solution was adjusted to desired value by stepwise addition of 2 M NaOH solution. The transmittance of the solution was recorded on a Shimadzu UV-1601 spectrophotometer using a quartz cell with an optical path length of 1 cm at 600 nm.

2.6. FITC labeling of chitosan

To label the chitosan with FITC (Onishi & Machida, 1999), chitosan was completely dissolved in 1% (v/v) acetic acid solution, then DMSO was added to form DMSO/1%HAc co-solvent system (75/25, v/v), and a predetermined amount of FITC in acetone was added. The reaction mixture was vigorously stirred for 24 h at room temperature. After reaction, the FITC-labeled chitosan was obtained through precipitation of the reaction mixture in excess acetone. Then, the FITC-chitosan was washed with acetone for 6 times. Finally, the FITC-chitosan was obtained by lyophilization.

2.7. In vivo chitosan test

Female mice (20–24 g body weight) were fasted for 12 h before the administration of chitosan. They were administered FITC-labeled chitosan solutions through an oral gavage tube that was carefully passed down through the esophagus into the stomach (Chae, Jang, & Nah, 2005). The FITC-chitosan solutions were prepared in 1% (v/v) acetic acid solution at a concentration of 21 g/l. The total volume of the administrated chitosan was 0.50 ml (at the dose of 500 mg/kg). To measure the absorbed amount of chitosan, the blood, liver, kidney, thymus, spleen, lung and heart were collected serially after the mice were sacrificed at predetermined time. The blood sample was directly mixed with 3.50 ml 0.5 M hydrochloride, and suspended for a night at room temperature. Other samples were homogenized by glass homogenizer, then 4.00 ml 0.5 M HCl was added. The solution was centrifuged at 2400 r/min for 20 min to separate the insoluble solid. Then, 0.50 ml the solution of sample was taken out and added to 3.50 ml phosphorous buffer solution (PBS, pH 6.86). The fluorescence intensity of the sample was measured using a fluorescence plate reader. The emission (EM) wave-

length was 520 nm and excitation (EX) wavelength was 495 nm. The obtained fluorescence intensities were normalized by the administered corresponding FITC labeled chitosan. Three mice were used for one dose of each sample. The plotted data are the mean ± *SD* ($n = 3$).

3. Results and discussion

3.1. M_w of chitosan samples

Fig. 1 shows the GPC profiles of chitosan samples. The shift towards to higher elution volumes was as a consequence of the reduction in average molecular weight. MCS and COS were obtained by degradation of the crude chitosan. The M_w of MCS and COS was 3.27×10^4 and 0.99×10^3, respectively. The COS mainly consisted of chitooligomers. The M_w, DDA and FTC content in the labeled chitosan samples were listed in Table 1.

3.2. Estimation of water-solubility

The water-solubility of chitosan was dependent on the pH of solution. Fig. 2 depicts pH dependence of water-solubility of chitosan. WSC was completely soluble at all tested pH. Most of COS molecules were water-soluble chitooligomers at all tested pH, and only a few molecules would precipitate at pH >8.5. HCS almost precipitated at pH >6.2, and most of MCS precipitated at pH 7.4. Thus, WSC and COS were completely soluble in acidic stomach, and also in intestine with pH 4.8–8.2 (Xiang, 2003). Some of HCS and MCS samples would gradually precipitate at the lower intestine if their chitosan molecules were not degraded into soluble molecules.

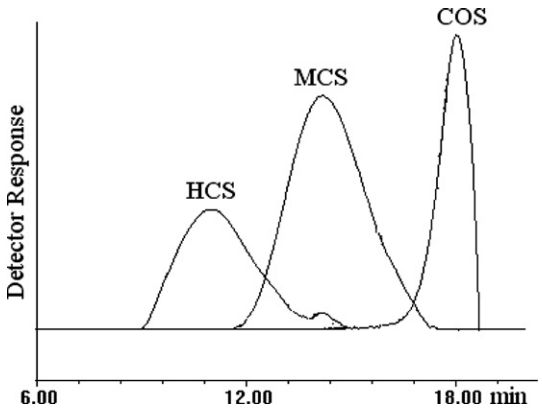

Fig. 1. GPC profiles of chitosan samples.

Table 1
Characterization of chitosan and FITC-labeled chitosan samples

Samples	DDA (%)	M_w	M_w/M_n	FITC content (%)
HCS	85.5	7.60×10^5	3.01	0.94
MCS	85.0	3.27×10^4	2.65	1.07
COS	85.9	0.99×10^3	1.07	1.22
WSC	52.6	3.91×10^4	2.78	0.98

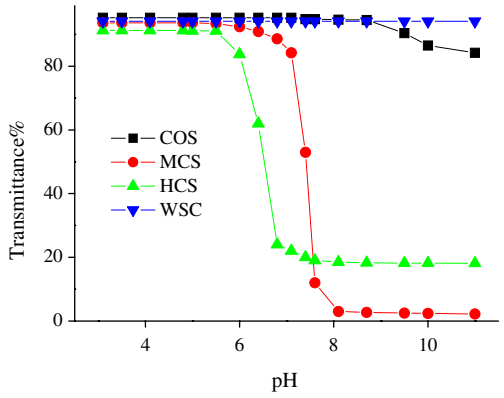

Fig. 2. pH dependence of water-solubility of chitosan.

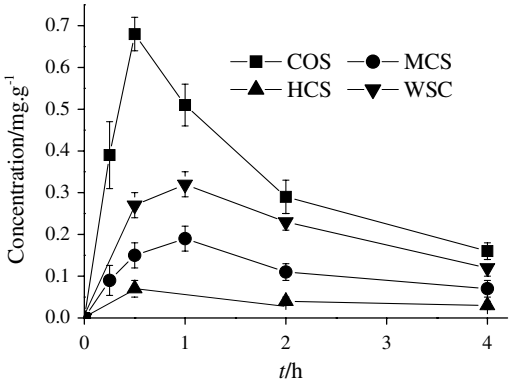

Fig. 3. Plasma chitosan concentrations after oral administration of chitosan samples.

3.3. Absorption of chitosan samples

Chae et al. (2005) reported that the concentration of the compound in blood reflected the intestinal absorption of the compound. Plasma chitosan concentrations were calculated on the basis of fluorescence intensity. These results are demonstrated in Fig. 3.

Intestinal absorption of chitosan was seriously affected by its molecular weight. At 0.5 h after oral administration, the absorbed amount of chitosan was in the order of HCS < MCS < COS, and COS showed much higher absorption than MCS and HCS. The result indicated that the intestinal absorption of chitosan increased with the decreasing of molecular weight.

The absorption profiles of MCS and WSC were similar. The plasma chitosan concentration increased in the first 1 h after administration, and then decreased, but the plasma chitosan level of WSC was higher than that of MCS at all tested times. MCS and WSC had the similar M_w, but WSC was water-soluble. The result indicated that good water-solubility of chitosan enhanced its intestinal absorption.

Both COS and WSC were soluble in intestine, but the plasma chitosan level of WSC was lower than that of COS at 0.5 h. The result also confirmed that the intestinal

absorption of chitosan with lower M_w was more rapid. The absorbed chitosan from COS rapidly distributed to other places in the body so that plasma chitosan level decreased rapidly. The results suggested that the absorbed chitosan might be water-soluble small molecules, the chitosan molecules were degraded to some extent before absorption.

In the case of HCS, plasma chitosan level was very low at 0.5 h and negligible later. The HCS could be dissolved in stomach. These soluble chitosan molecules could be degraded by the enzymes in upper intestine, but the dissolved macromolecules would gradually precipitate with increasing pH in lower intestine, which made the chitosan difficult to be further degraded by the enzymes and was hardly absorbed by intestine. A large quantity of chitosan with high molecular weight had been found in feces when it was utilized as dietary fiber supplements.

3.4. Distribution of absorbed chitosan in organs

In the present work, the distribution of chitosan in organs was checked by the oral administration. At predetermined time after administration, the liver, kidney, spleen, heart, lung and spleen were examined for the chitosan concentration.

Liver was the first barrier for xenobiotics after xenobiotics were absorbed (Xiang, 2003). Therefore, the concentration of chitosan in liver was an important index to reflect absorption. The chitosan concentration in liver was shown in Fig. 4. The chitosan level in liver was much higher than in plasma, indicating that absorbed chitosan had some accumulation in liver. In case of WSC, the maximum liver chitosan concentration was at 2 h when the maximum plasma chitosan concentration was at 1 h. The liver chitosan concentration from MCS increased during the tested 4 h, and was lower than that of WSC at the all tested times. Of course, the liver chitosan concentration from MCS would decrease after 4 h (Onishi & Machida, 1999; Tai, Sheu, Lee, Yao, & Chaiang, 2000).

Interestingly, among the four chitosan samples, at 0.50 h, COS had the lowest liver chitosan concentration but the highest plasma chitosan concentration, and HCS had the highest liver chitosan concentration but the lowest plasma chitosan concentration. The liver chitosan concentration from COS and HCS decreased within 0.5–4.0 h. The results suggested that the absorbed chitosan molecules from COS were very small molecules, and the absorbed chitosan molecules from HCS were larger molecules. The larger chitosan molecules were subject to rapider plasma clearance, and had higher liver accumulation level.

From 2 to 4 h, the liver chitosan concentration from HCS decreased while that from MCS still increased, indicating that HCS and its degraded product was precipitated earlier than MCS and its degraded product in intestine. The results confirmed that the precipitated chitosan macromolecules was difficult to be further degraded by the enzymes, and was hardly absorbed by intestine.

Figs. 5–8 show the chitosan concentration in the tested organs from the four labeled chitosan samples, respectively. The absorbed chitosan from the four samples could be distributed to all tested organs during the period.

The total concentration of chitosan from WSC in all organs maintained high from 0.5 to 4.0 h. The concentration of chitosan from COS in the organs was generally lower than that of other samples. It could be explained by the fact that chitooligomer was quickly absorbed by intestine and easily penetrated these bio-membranes to reach other tissues, enter body fluids and be excreted in urine (Onishi & Machida, 1999; Richardson, Kolbe, & Duncan, 1999). It indicated that chitooligomer by p.o. had no significant accumulation effect in the body.

3.5. Discussion

The M_w and water-solubility of chitosan can be considered as a critical parameter for the absorption of chitosan in mice after p.o. As the molecular weight increased, the absorbed amount of chitosan decreased. It could be due to M_w-dependent transport phenomena (Chae et al., 2005). Chitooligomer was easily absorbed by intestine, which was confirmed by confocal laser microscopy. In the case of FITC-labeled water-soluble chitosans, dense

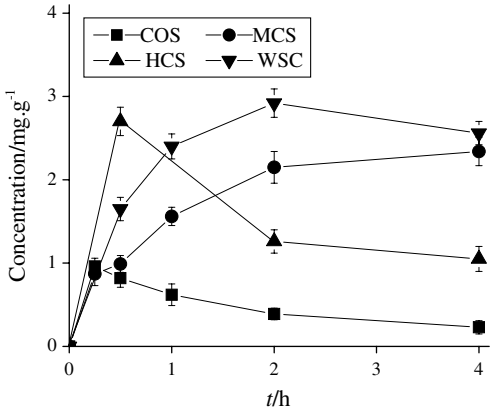

Fig. 4. Liver chitosan concentration after oral administration of chitosan samples.

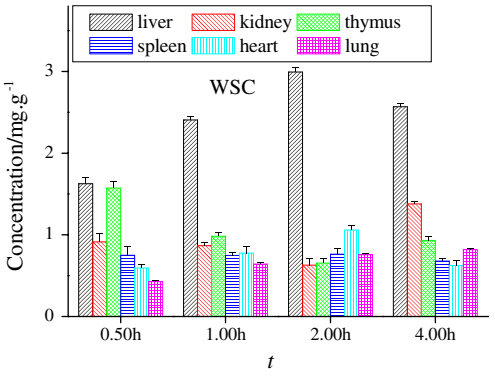

Fig. 5. Concentration of chitosan in organs after oral administration of FITC-WSC.

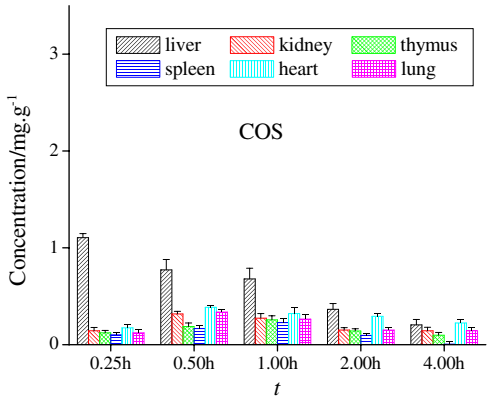

Fig. 6. Concentration of chitosan in organs after oral administration of FITC-COS.

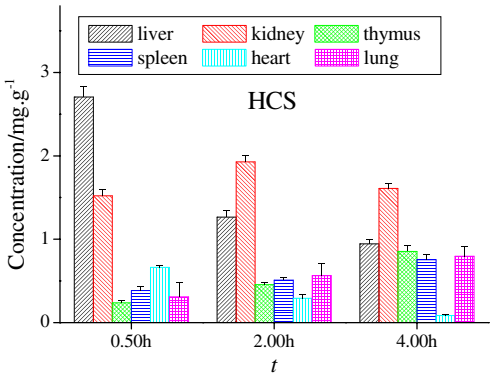

Fig. 7. Concentration of chitosan in organs after oral administration of FITC-HCS.

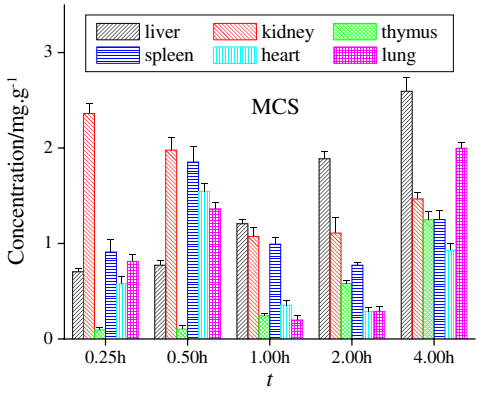

Fig. 8. Concentration of chitosan in organs after oral administration of FITC-MCS.

fluorescence intensity was observed at the epithelium of villi in duodenum and jejunum compartment. The fluorescence intensity decreased with the increasing M_w of chitosan.

Another possible reason was that the membranes just permit chitosan with a certain size (less than 60 kDa) to pass through (Xiang, 2003). Therefore, the absorbed amount of chitosan molecules was less than the administered amount. The intestinal absorption of chitosan in vivo was also influenced by their water-solubility. Apparently, solid transport of chitosan is difficult while dissolved chitosan in fluid can be easily absorbed by intestinal.

Absorbed chitosan was distributed to all tested organs such as liver, kidney, blood, spleen and so on. The concentration of chitosan in blood was much less while the concentration in liver was much higher. It was associated with particularly rapid plasma clearance after absorption. Richardson et al. (1999) found that ^{125}I-labeled chitosan samples ($M_w > 5$ kDa) were subject to particularly rapid plasma clearance after intravenous injection.

It was interesting that three chitosan samples (HCS, MCS and WSC) showed high liver and kidney distribution after oral administration. The kidney was a main excretion manner, and it just excrete xenobiotics with molecular weight lower than 500 (Xiang, 2003). The liver was the other main excretion manner and the first guard barrier for body when xenobiotics entered body, and it excreted metabolite with M_w between 500 and 5000 (Xiang, 2003). Chitosan with high molecular weight showed high liver distribution after oral administration. Chitosan was reported to excrete into urine after i.v. (Onishi & Machida, 1999). It indicated that chitosan was quickly eliminated from the body. The quick elimination of chitosan from the body was considered to be probably due to its extensive biodegradability. Thus, we concluded that chitosan underwent the enzyme-catalyzed degradation in the body to become the products with uniformly fairly small molecular size. It was in agreement with the reported results about biodegradation and distribution of water-soluble chitosan in mice. Intestinal absorptions of chitosan by oral administration were highly influenced by its molecular weight and its water-solubility.

The physiological activity of chitosan was correlation to its absorption and body distribution. The cholesterol-lowering effect of chitosan is extensively studied recently. The chitosan with middle molecular weight (5–50 kDa) was effective, but the chitosan (>300 kDa) and chitooligomers (<2 kDa) was less effective as hypocholesterolemiant (Sugano, Watanabe, Kishi, Izume, & Ohtakara, 1988). The 20 kDa chitosan prevents progression of diabetes mellitus and exhibits higher affinity for lipopolysaccharides than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). Chitosan with very high molecular weight was very difficult to be absorbed and enter the blood. Chitooligomers was easily degraded into much smaller molecules, quickly absorbed and distributed to other places. The chitosan like MCS could be degraded and absorbed after p.o. and the molecules of absorbed chitosan were not very small, which was necessary for cholesterol absorption in blood.

Water-soluble chitosan with higher degree of polymerization had better antitumor effect (Qin et al., 2002; Tokoro et al., 1988). The mechanism of action involved enhancement of the immunological system in the host animal. The spleen and thymus are the important immune organs. The chitosan level in spleen and thymus from WSC kept

much higher than that from COS and HCS. Chitooligomers were easily absorbed, penetrated bio-membranes and distributed to body liquids and other tissues. With the amino groups, chitooligomers could regulate acid–base balance of body liquids.

Chitosan with high M_w was rarely absorbed in that it became gel and precipitated when the pH of the intestine was neutral or alkaline. The chitosan gel imbedded fat in intestinal so that fat could not be absorbed, and greatly increases fecal fat excretion (Gades & Stern, 2003). The high dose of chitosan with high M_w could contribute to weight loss, but low dose of chitosan did not have noticeable effect of weight loss.

4. Conclusion

Absorption of chitosan by intestine was influenced by its molecular weight and water-solubility. The absorption of chitosan increased with the decrease of M_w and the increase of water-solubility. After chitosan molecules were absorbed, they were distributed to these tested organs such as liver, kidney, spleen, and thymus and so on. The different chitosan should have different physiological activities. Water-soluble chitosan WSC was maintaining a much high concentration in these tested organs for a long time, suggesting that WSC was a good candidate as drug carrier for lasting target drug delivery system.

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